Efficacy of Anti-Endotoxin Monoclonal Antibody E5 Alone or in Combination with Ciprofloxacin in Neutropenic Rats with *Pseudomonas* Sepsis

Rodrigo L. C. Romulo, John E. Palardy, and Steven M. Opal

Division of Infectious Diseases, Brown University and Memorial Hospital, Providence, Rhode Island

Pathogen free rats were rendered neutropenic, given oral feedings of *Pseudomonas aeruginosa* 12.4.4, then monitored for fever. At the onset of fever, rats were given intravenous treatment with either anti-endotoxin monoclonal antibody (MAb) E5 or control MAb B55. Survival was significantly greater in E5- than in B55-treated animals ($P < .01$). Serum levels of both lipopolysaccharide and tumor necrosis factor-α were significantly reduced in E5- versus B55-treated rats 24 h after treatment ($P < .01$ and $< .05$, respectively). Rats were also treated with E5 or B55 in combination with a suboptimal dose of ciprofloxacin at fever onset and again 24 h later. Survival was significantly greater in ciprofloxacin-treated animals given E5 than in animals given B55 ($P < .005$). Posttreatment endotoxin levels were decreased in animals receiving E5 in combination with ciprofloxacin ($P < .001$) compared with B55-treated animals. These results indicate that therapy with anti-endotoxin MAb E5 alone or in combination with antimicrobial therapy improves survival in this bacteremic infection model of *Pseudomonas* sepsis.

Mortality from gram-negative sepsis and bacteremia remains unacceptably high despite the availability of potent broad-spectrum antimicrobial agents [1]. Cancer patients rendered neutropenic by cytoreductive chemotherapy are at a particularly increased risk of death from this infection [2]. Recently published prospective, double-blind, placebo-controlled, multicenter trials have demonstrated the potential efficacy of therapy with monoclonal antibody (MAb) directed against the core glycolipid component of endotoxin in the treatment of gram-negative bacteremia and septic shock [3, 4]. However, only small numbers of neutropenic patients were included in these trials. McCutchan et al. [5] showed that neutropenic patients given single prophylactic intravenous doses of polyclonal *Escherichia coli* J5 antiserum were not protected from gram-negative infections. No clinical studies to date have attempted to use anti-endotoxin antibodies as an adjunct to the treatment of actual gram-negative infections in neutropenic patients.

Animal studies have also demonstrated, albeit inconsistently, that antibodies to core glycolipid can be protective in experimentally induced gram-negative sepsis [6–9]. Most animal models using endotoxin challenge or live bacterial challenge with large numbers of organisms introduced through the intravenous, intraperitoneal (ip), or subconjunctival routes do not replicate the events that actually occur clinically [10–12]. A rodent model of antineutropenic agent use has been developed that closely mimics the pathophysiologic changes taking place in a febrile neutropenic cancer patient [13]. This infection model reproducibly induces *Pseudomonas* bacteremia and multiorgan failure in the experimental animals. The present study was done to determine whether survival is enhanced by treatment with an anti–core glycolipid MAb alone or in combination with an antimicrobial agent in neutropenic rats with experimental *Pseudomonas* septicemia.

Materials and Methods

Pharmacologic agents. Injectable cyclophosphamide (cyclophosphamide monohydrate; Sigma, St. Louis) and injectable cefamandole (Mandol; Eli Lilly, Indianapolis) were obtained commercially. Both were diluted in sterile normal saline. Injectable ciprofloxacin (Cipro; Miles Pharmaceuticals, West Haven, CT) was also obtained commercially. A murine-derived IgM MAb directed against the lipid A component of bacterial endotoxin, E5 [14, 15], was provided by Xoma (Berkeley, CA). This antibody has previously been reported to possess potent anti-endotoxin activity in experimental animals [12]. An IgM MAb, B55 (also provided by Xoma), was used as an isotype control. B55 is an anti–breast carcinoma MAb [16].

Bacteria. The test organism, *Pseudomonas aeruginosa* 12.4.4, belongs to Fisher-Devlin-Gnabasik immunotype VI and is a serum-resistant, virulent human isolate (provided by A. McManus, US Army Institute of Surgical Research, San Antonio, TX). The organism was stored in whole sheep's blood agar (Adam Scientific, West Warwick, RI) at −70°C until use. The day before oral challenge, the isolate was inoculated in trypticase soy broth (TSB; Becton Dickinson, Cockeysville, MD) and incubated overnight at 37°C. The next day, bacteria were suspended in sterile normal saline and adjusted spectrophotometrically to 10^9 organisms/mL.

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Reprints or correspondence: Dr. Rodrigo L. C. Romulo, Division of Infectious Diseases, Memorial Hospital of Rhode Island, 111 Brewster St., Pawtucket, RI 02860.

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Animal model. Ninety-two female, albino, pathogen-free Sprague Dawley rats weighing 120–135 g (Charles River Laboratories, Wilmington, MA) were maintained in clear polypropylene cages (4 animals/cage) covered with high-efficiency particulate filter covers to prevent cross-contamination or exogenous infection of the animals. The animals were given cefamandole intramuscularly at a dose of 100 mg/kg at 4 days, 2 days, and 1 day before oral bacterial challenge. Previous experience has shown that this produces no ill effects in nonneutropenic animals. Oral bacterial innoculations were given in a total volume of 1 mL per animal via soft polyethylene tubing that was free of tissue reaction (Intramedic PE-160; Becton-Dickinson, Parsippany, NJ). The time of the first bacterial challenge was designated time 0, and two more innoculations were given 48 and 96 h later. Cyclophosphamide was given at a dose of 150 mg/kg ip at time 0 and a second dose of 50 mg/kg ip at 72 h. This consistently resulted in a period of profound neutropenia within 3 days that lasted ~5–7 days [13, 17].

Before onset of neutropenia, a 5- × 5-cm area of fur was shaved off the rats to allow for accurate and repeated body temperature readings. A noncontact infrared digital thermometer (Markson Science, Phoenix) was used to monitor the animals' body temperatures several times daily. All manipulations were done under light CO_2 (dry ice) anesthesia.

MAb and antimicrobial treatment. At the onset of fever, animals received monotherapy with either MAb E5 (24 animals) or MAb B55 (14 animals), given as a single intravenous dose (2 mg/kg). No antimicrobial agent was given to these animals. Additional experiments were done with animals empirically treated with a combination of either oral ciprofloxacin or oral saline and an intravenous dose of either the anti-endotoxin MAb E5 or the control MAb B55 (each, 2 mg/kg). A suboptimal dose of ciprofloxacin (1.25 mg/kg) was chosen on the basis of prior experiments that demonstrated that this dose offers a protective effect in ~50% of neutropenic animals [13]. The oral ciprofloxacin doses were given every 12 h for 3 days. Two single daily doses of either MAb were given. Twenty-two animals received MAb E5 and ciprofloxacin, 22 received B55 and ciprofloxacin, and 10 received saline and control MAb B55.

Blood and necropsy studies. Blood samples for quantitative culture and endotoxin levels were taken from the retroorbital plexus of each animal 5 days before oral feeding with the challenge strain, at the onset of fever, and 24 h after the first dose of antibody therapy. Animals were examined daily and deaths recorded. Necropsies were done within 24 h on rats that died, and bacterial cultures from heart, liver, lung, spleen, and cecal tissue were obtained. Blood culture specimens were serially diluted in normal saline and then plated on MacConkey's agar and incubated for 24 h at 37°C. Oxidase-positive non-lactose-fermenting isolates were then further identified as the challenge strain with a polyvalent *P. aeruginosa* antisera set (Difco, Detroit). *Pseudomonas* isolates of identical serotype were interpreted as evidence of systemic infection from the oral challenge strain of *P. aeruginosa* 12.4.4.

Quantitative colony counts of liver and splenic tissue were done on each animal that did not survive the experimental period. Tissue samples were weighed, minced, and serially diluted in TSB and then directly plated on MacConkey's agar plates.

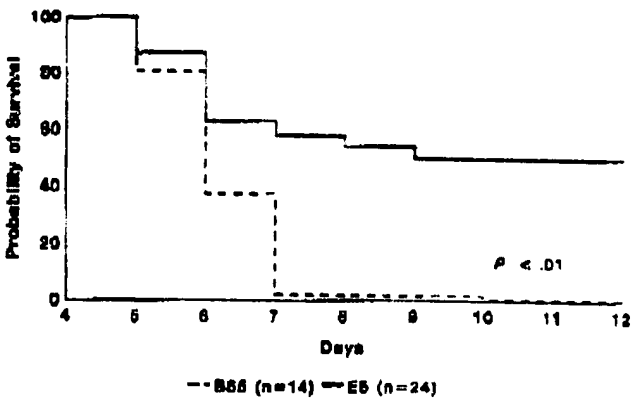

Figure 1. Kaplan-Meier survival estimates of neutropenic rats with *Pseudomonas* sepsis treated with either anti-endotoxin monoclonal antibody (MAb) E5 or isotype control MAb B55 without antimicrobials.

Circulating levels of bacterial endotoxin were measured by quantitative limulus lysate assay (Associates of Cape Cod, Woods Hole, MA) on serum taken 5 days before oral feeding, at the onset of fever, and 24 h after starting empiric therapy. The endotoxin standard used was *E. coli* O113 (Associates of Cape Cod) prepared in both endotoxin-free serum and endotoxin-free water. The lower limit of detection of the assay was 0.001 endotoxin units (EU)/mL. Tumor necrosis factor-α (TNFα) levels were determined using an L929 cytotoxicity assay [17] in animals receiving monotherapy with either E5 or B55.

Statistical analysis. Differences in survival between treatment groups were analyzed by Kaplan-Meier survival curves and compared by Wilcoxon statistic. Blood determinations and endotoxin levels were analyzed by two-tailed Student's *t* test. Results are expressed as mean ± SD; differences were considered significant at $P < .05$.

Results

Survival. Animals were uniformly febrile within 5 days of the initiation of cyclophosphamide therapy. Control animals became overtly ill (inactivity, poor feeding, piloerection) and failed to survive beyond 72 h after the onset of fever. Animals treated with the anti-endotoxin MAb E5 had a significantly greater likelihood of survival over the 10-day study period than did animals treated with MAb B55 (figure 1). A total of 12 of 24 E5-treated animals survived while none of the 14 control animals survived ($P < .01$).

Ciprofloxacin treatment at fever onset marginally improved the outcome in neutropenic animals with 6 (27%) of 22 surviving with B55 and ciprofloxacin therapy, and none of 10 surviving with saline and B55 treatment ($P = .08$). However, the addition of MAb E5 in combination with ciprofloxacin yielded the greatest survival rate: 17 (77.3%) of 22 animals (figure 2).

Blood cultures and necropsy findings. During the period

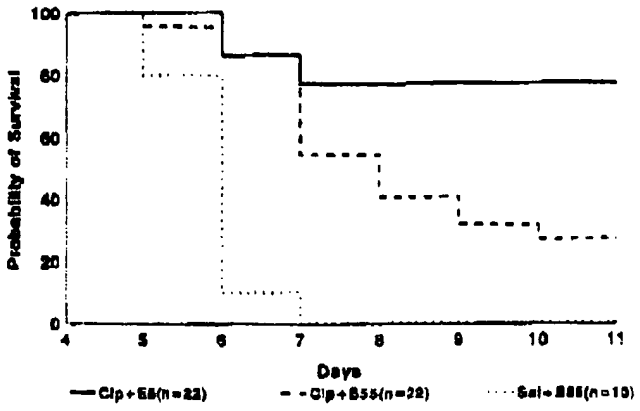

Figure 2. Kaplan-Meier survival estimates of neutropenic rats with *Pseudomonas* sepsis treated with anti-endotoxin monoclonal antibody (MAb) E5 and ciprofloxacin (Cip), isotype control MAb B55 and Cip, and MAb B55 and saline (Sal).

of neutropenia, a total of 96.3% of all animals had documented bacteremia with the challenge strain of *P. aeruginosa*. In the absence of ciprofloxacin therapy, the frequency of bacteremia did not differ between animals treated with E5 (21/22) and those given the control B55 (21/22). Although the E5-treated animals actually had slightly higher quantitative levels of bacteremia (134 ± 93 cfu/mL) than did the B55-treated animals (92 ± 52 cfu/mL), the difference was not significant. These results show that E5 did not clear bacteremia in these animals. In contrast, animals treated with ciprofloxacin showed lower quantitative levels of bacteremia (116 ± 53 cfu/mL [n = 44]) than those receiving oral saline (371 ± 163 cfu/mL [n = 10], P = .03). Multiorgan infection was demonstrated by tissue culture in 4 of 5 deaths in the group treated with MAb E5 and ciprofloxacin, in 15 of 15 deaths in the MAb B55 and ciprofloxacin treatment group, and in 7 of 10 deaths in the group given MAb B55 and saline. The colony counts of hepatic and splenic tissue cultures were not significantly lower in the E5-treated animals than in the B55-treated animals (330 ± 1179 cfu/mL versus 812 ± 2059 cfu/mL, P = .2).

Endotoxin and TNF levels. No circulating free endotoxin was detected in baseline samples from animals before initiation of the experimental protocol. In the animals receiving only E5 or B55, endotoxin levels did not differ at the onset of fever but posttreatment levels were significantly lower in the E5-treated group than in the MAb B55 group (0.68 ± 0.24 EU/mL vs. 6.4 ± 2.1 EU/mL, P < .01). TNFα levels measured by cytotoxicity assay paralleled the endotoxin levels with no significant difference observed in levels before treatment and at fever onset, but the E5 group had significantly lower TNFα levels 24 h after therapy (48 ± 3.7 pg/mL vs. 433 ± 68.4 pg/mL, P < .05). For animals receiving ciprofloxacin, the mean endotoxin levels at the onset of fever in animals who subsequently received E5 or B55 did not differ (1.74 ± 0.501 vs. 3.21 ± 2.02 EU/mL, P = .44); however, the mean serum endotoxin level was significantly lower after the first dose of E5 (0.42 ± 0.52 EU/mL) than after B55 (7.37 ± 1.75 EU/mL, P = .001; figure 3).

Discussion

Endotoxin, a lipopolysaccharide (LPS) component of gram-negative bacterial cell walls, is known to initiate the complex cascade of events resulting in septic shock [18]. Normal human subjects given an intravenous dose of endotoxin developed not only functional changes similar to those observed in septic shock (increased cardiac index and heart rate and decreased systemic vascular resistance and blood pressure) but also depression of left ventricular function that was independent of changes in left ventricular volume or vascular resistance [19].

The use of MAbs to endotoxin represents a major advance in the management of the septic patient with gram-negative bacterial infection [3, 4]. Anti-endotoxin antibodies appear to function by disrupting endotoxin activity (i.e., suppressing or diminishing the release of mediators such as cytokines) without promoting opsonophagocytosis or clearance of gram-negative bacteria from the bloodstream [6, 12]. The results of the current study support this view in that the level of gram-negative bacteremia was not affected by the anti-endotoxin antibody, yet the level of biologically active endotoxin in the blood was diminished and survival was enhanced by antibody treatment.

MAb E5 is a murine IgM monoclonal that reacts with the lipid A component of bacterial endotoxin [14, 15]. Since lipid A is common to virtually all endotoxins found in gram-negative bacteria, E5 has the potential for cross-protective

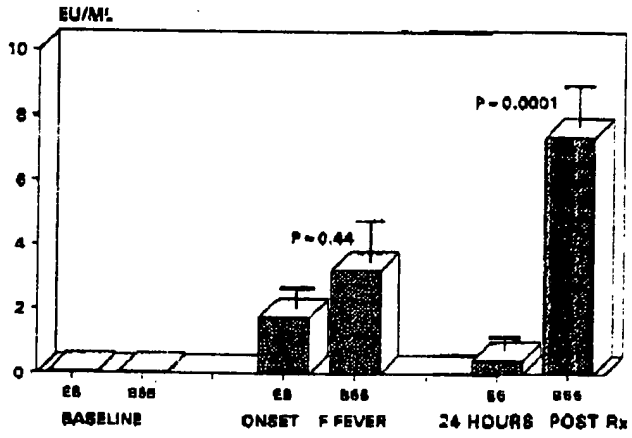

Figure 3. Endotoxin levels in neutropenic rats with *Pseudomonas* sepsis receiving ciprofloxacin and either anti-endotoxin monoclonal antibody (MAb) E5 or control MAb B55 before and after onset of fever and 24 h after start of empiric therapy. EU = endotoxin units, Post Rx, after MAb.

activity. Most wild-type gram-negative bacteria synthesize smooth-form LPS that have O-specific polysaccharide chains attached to the lipid A core oligosaccharide. In vitro, E5 has been shown to bind a diverse spectrum of smooth LPS preparations from clinically relevant wild-type gram-negative bacteria including *Escherichia, Klebsiella, Proteus, Pseudomonas, Salmonella, Serratia,* and *Yersinia* species [15]. LPS isolated from wild-type organisms usually contains significant amounts of rough LPS. E5 has likewise been shown to bind an extensive panel of rough LPS preparations [14]. In a recent clinical study [4], E5 was demonstrated to possibly enhance resolution of organ failure if given to patients with gram-negative sepsis before the development of shock. However, the ability to prevent death in human patients was not confirmed in the second placebo-controlled trial of E5 [20, 21].

Recent clinical studies have pointed out the significance not only of the presence of detectable endotoxemia but also of its concentration. In a study investigating endotoxemia in humans with septic shock, Danner et al. [22] found that endotoxemia occurred commonly, though intermittently, and was of clinical importance. Endotoxemic patients were more likely to develop renal insufficiency, acute respiratory distress syndrome, and cardiac depression than were nonendotoxemic patients. Schedel et al. [23] recently showed that in patients with septic shock treated with a polyclonal human immunoglobulin preparation directed against the core glycolipid of endotoxin in addition to antibiotics, survivors had significantly lower plasma endotoxin levels than did nonsurvivors in the first 24 h after development of septic shock. In our study, the administration of anti-endotoxin MAb to rats with *Pseudomonas* sepsis significantly diminished the quantitative level of circulating free endotoxin and improved outcome.

Reduction in circulating endotoxin levels decreases the stimulus for the generation of essential proinflammatory cytokines, such as TNFα, from the monocyte/macrophage cell line [24, 25]. Since the generation of proinflammatory cytokines has been shown to be important in the pathophysiology of septic shock [24–28], reduction of these cytokines with an anti-endotoxin antibody may provide an explanation for their mechanism of protection. Results of our study are supportive of such a hypothesis. TNFα levels were significantly lower in animals treated with the anti-endotoxin antibody E5 than in control animals. This is further supported by a recent study showing inhibition of TNFα production by MAbs to LPS (from rough J5 mutant *E. coli* O111:B4) in mice experimentally infected with *E. coli* [29].

While there is clinical evidence to support the efficacy of anti-endotoxin MAb in the treatment of sepsis [3, 4], a number of experimental and animal studies have failed to demonstrate a beneficial effect from anti-endotoxin immunotherapy [8, 9, 30]. An explanation for the variable and often conflicting experimental results may relate to the nature of the test conditions, animal models [8, 9], and antibody preparations [8, 30] used in such studies. We used a rigorous, clinically relevant model of an actual infection with a virulent gram-negative bacterium in immunocompromised animals [13, 17]. This model replicates many of the pathophysiologic events that occur in febrile neutropenic cancer patients. The animals become septic from an endogenously mediated bacteremia across mucous membranes. The infecting organism is a viable, serum-resistant, actively replicating, pathogenic microorganism that is presented to the test animal in a manner similar to that by which most patients become septic. No therapeutic agent, either anti-endotoxin MAb or antimicrobial agent, was given until the animals became septic as manifested by onset of fever.

The present study shows improvement in animal survival when E5 is used alone or when two doses of E5 are given in combination with ciprofloxacin, a fluoroquinolone antibiotic effective against *P. aeruginosa*. In a recent study using a neutropenic mouse model of *Pseudomonas* pneumonia, the combination of serotype-specific MAbs directed against the O side chain of LPS and the newer fluoroquinolone sparfloxacin similarly increased survival when compared to each agent used alone [31]. Although MAb E5 did not affect the frequency or level of bacteremia or multiorgan infection in treated animals in our experiment, its administration resulted in diminished levels of circulating endotoxin. Endotoxin levels have previously been shown by Shenep et al. [32] to be elevated after antibiotic therapy of gram-negative sepsis. In this study there was a demonstrable reduction in quantitative endotoxin levels after administration of E5 despite concomitant administration of an antimicrobial agent. This reduction in endotoxin level may explain the increased survival observed in the animals receiving E5. The treatment regimen used in this study is similar to the empiric therapy potentially available to febrile neutropenic cancer patients in the near future. The results of this study suggest that anti-endotoxin MAb may be a beneficial adjunct to conventional antimicrobial agents in the treatment of neutropenic cancer patients with gram-negative sepsis.

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